Direct neutralization of alkaline-denatured plasmid DNA in sequencing protocol by the sequencing reagent itself

Evelyne Rouer
ICGM, INSERM U-332, 22 Rue Méchain, 75014, Paris, France

Received July 14, 1994; Revised and Accepted September 21, 1994

Denaturation of the double stranded DNA is required before sequencing by the dideoxy chain termination method (1). Good results are routinely obtained with the alkaline-denaturation method, which involves incubation of the DNA in the presence of 0.2 M NaOH for a few minutes at room or higher temperatures (2,3). The alkaline mixture is then neutralized by the addition of sodium acetate (0.3 M), followed by ethanol precipitation to remove the excess salt. Taking into account the DNA loss during these steps, amounts of 3 to 5 μg of template are generally used. Thus these steps are both DNA and time consuming. An interesting modification to this original protocol, based upon the principle of neutralization of 1 volume of 1 N NaOH by 1 volume of 1 N HCl, has been reported which allows the ethanol precipitation to be bypassed (4).

Here we report that neutralization of the alkaline mixture does not require any addition of salt or acidic solutions, since a reagent with a good buffer capacity is used later in the sequencing protocol. We obtained satisfactory sequencing data with different plasmid DNA preparations in the conditions described below.

The plasmid DNA was prepared on Quiagen Tip 100, from a 30 ml over-night culture in LB medium plus ampicillin (50 mg/ml) of DH5α bacteria transformed with a recombinant plasmid pTZ18 containing a fragment of *Ick* cDNA (5). Quantification of the DNA solution was performed at 260 nm. Sequencing was performed with the Sequenase kit (USB). Plasmid DNA (2 μg) was denatured with 1 μl of 0.1 M NaOH in 4 μl total volume for 5 min at 37°. Then 2 μl of the 5× sequencing buffer are added with 4 μl of the universal primer (2 pmoles),and the annealing is followed for 5 min at 37°. During this time a mixture containing 2 μl of dGTP mix (five fold diluted), 1 μl of 0.1 M DTT, 0.5 μl to 1 μl of α^{33}P dCTP (10 mCi/ml, Amersham) and 2 μl of Sequenase enzyme (eight fold diluted), is prepared. This mixture (6 μl) is added and the polymerisation is performed at room temperature for 2 to 5 min. The remaining steps were exactly as described in the USB booklet. We observed that DNA sequencing can be performed with a low amount of template, as low as 0.5 μg. The data shown in the figure were respectively obtained with 2 μg (A) and 0.5 μg (B) of *Ick* plasmid DNA. As a comparison, we also show the result obtained using 2 μg of *Ick* plasmid DNA and the salt solution neutralization step (C), as indicated in the Sequenase booklet. We obtained similar good sequencing data with another plasmid DNA (pGEX-2T) using GST specific primers (Pharmacia) (data not shown).

The composition (1×) of the sequencing buffer is 40 mM Tris−HCl pH 7.5, 20 mM $MgCl_2$ and 50 mM NaCl. Because of its great buffer capacity, we think that a Tris solution is better and safer than an acid solution to neutralize the alkaline DNA mixture.

REFERENCES

1. Sanger,F., Nicklen,S. and Coulson, A.R. (1977) *Proc. Natl Acad. Sci. USA* **74**, 5463−5467.
2. Chen,E.J. and Seeburg,P.H. (1985) *DNA* **4**, 165−170.
3. Lim,H.M. and Pene,J.J. (1988) *Gene Anal. Techniques* **5**, 32−39.
4. Hsiao Ku-chuan (1991) *Nucleic Acids Res.* **19**, 2787.
5. Rouer,E., Van Huynh,T., Lavareda de Souza,S., Lang,M.C., Fischer,S. and Benarous,R. (1989) *Gene* **84**, 104−113.

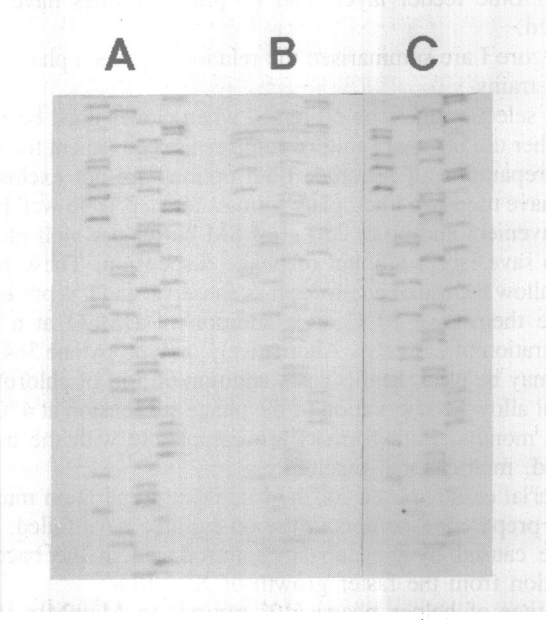

Figure 1. Sequencing of the plasmid *Ick* cDNA as described in the text, with either 2 μg (A) or 0.5 μg (B) of template. (C) Comparison with the standard protocol of neutralization (salt solution) (2 μg of template). Lanes from left to right are A,G,C,T.